ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2000, p. 1694–1696 Vol. 44, No. 6
0066-4804/00/$04.00+0

In Vitro Susceptibility Tests for Cationic Peptides: Comparison of Broth Microdilution Methods for Bacteria That Grow Aerobically

A. GIACOMETTI,* O. CIRIONI, F. BARCHIESI, M. S. DEL PRETE, M. FORTUNA, F. CASELLI, AND G. SCALISE

Institute of Infectious Diseases and Public Health, University of Ancona, Italy

Received 13 September 1999/Returned for modification 7 January 2000/Accepted 26 February 2000

The in vitro susceptibilities of 90 clinical isolates of gram-positive and gram-negative aerobic bacteria to six cationic peptides, buforin II, cecropin P1, indolicidin, magainin II, nisin, and ranalexin, were evaluated by two broth microdilution methods. The first method was performed according to the procedures outlined by the National Committee for Clinical Laboratory Standards for bacteria that grow aerobically, while the second was performed according to the procedures recently proposed by the R. E. W. Hancock laboratory for testing antimicrobial peptides. Overall, the first method produced MICs two- and fourfold higher than the second method.

Cationic peptides have been isolated from various biological sources (2–6, 10, 11–13, 19). In mammals, including humans, they are found in the neutrophil and on the surface of the tongue, trachea, lungs, and upper intestine. In fact, cationic peptides are thought to be major factors in antibacterial defense on mucosal surfaces (10, 11), and because of their antimicrobial potency they may have therapeutic potential in the treatment of infections (1, 6–10, 12–14, 16–18). Many of these compounds carry net positive charges, and it has been suggested that their mode of action as antimicrobial agents may be similar and may involve the formation of ion channel pores spanning the membranes without requiring a specific target receptor (10, 11, 16). Nevertheless, since several peptides have a tendency to precipitate and bind avidly to the surface of target cells or plastic materials, such as polystyrene, methods for evaluating the in vitro antimicrobial activities of these compounds are debated (10, 11). The main aim of this study was to compare two different broth microdilution methods to evaluate the antimicrobial activity of the cationic peptides: the first was performed according to the procedures outlined by the National Committee for Clinical Laboratory Standards (NCCLS) for bacteria that grow aerobically (15), while the second was based on the procedures recently proposed by R. E. W. Hancock (University of British Columbia, Vancouver, British Columbia, Canada) for testing antimicrobial peptides (http://www.interchg.ubc.ca/bobh/MIC.htm). Secondarily, time-kill kinetics were determined to point out the influence of polypropylene and polystyrene in bactericidal activity.

A total of 90 nonduplicate, clinical isolates were tested and were found to consist of methicillin-susceptible *Staphylococcus aureus* (30 strains), *Pseudomonas aeruginosa* (30 strains), and *Escherichia coli* (30 strains).

Buforin II, cecropin P1, magainin II, indolicidin, nisin, and ranalexin were obtained from Sigma-Aldrich S.r.l. (Milan, Italy). The aminoglycoside amikacin (Sigma-Aldrich) was used as a control cationic antimicrobial agent. The drugs were dissolved in distilled water. Solutions were made fresh on the day of assay or stored at −80°C in the dark for short periods. The MIC of each peptide was determined by two broth microdilution methods with cation-adjusted Mueller-Hinton (MH) broth (Becton Dickinson Italia, Milan, Italy) and an initial inoculum of 5×10^5 CFU/ml.

The first method was performed according to the procedures outlined by the NCCLS (15). Polystyrene 96-well plates (Becton Dickinson and Co., Franklin Lakes, N.J.) incubated for 18 h at 35°C in air. The MIC was considered the lowest drug concentration at which observable growth was inhibited. The minimal bactericidal concentration (MBC) was considered the lowest concentration of each drug that resulted in a >99.9% reduction in CFU of the initial inoculum.

The second method was performed according to the procedures recently proposed for testing antimicrobial peptides by R. E. W. Hancock. Since cationic peptides bind polystyrene, polypropylene 96-well plates (Sigma-Aldrich) were substituted for polystyrene plates and incubated for 18 h at 37°C in air. The MIC was considered the lowest drug concentration that reduced growth by more than 50% compared with the growth in the control well. The viable count in each well was determined by performing 10^{-6} dilutions and plating 10 μl of each dilution onto MH agar plates to obtain overnight cultures. The MBC was determined by plating out the contents of wells that showed no visible growth of bacteria onto MH agar plates and incubating at 37°C for 18 h. The MBC was considered the lowest concentration of each drug that prevented any residual colony formation.

S. aureus ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were used as quality control strains. In all experiments, MBC assays were performed by diluting the samples in sodium HEPES buffer (pH 7.2) to minimize the carryover effect, to halt the peptide killing at the final sampling time, and to avoid peptide-induced organism clumping. Experiments were performed in triplicate. The significance of differences was evaluated by Student's *t* test for paired samples. A *P* value of ≤0.05 was considered significant.

Overall, when the polycationic peptides were tested, the first method produced MICs and MBCs usually fourfold higher than the second method. Actually, MICs and MBCs obtained by using the NCCLS method were significantly higher than those produced by the Hancock method ($P < 0.001$). On the other hand, although some differences were observed when the control agent amikacin was tested, these were not statistically

* Corresponding author. Mailing address: Clinica delle Malattie Infettive, c/o Azienda Ospedaliera Umberto I, Piazza Cappelli, 1, 60121 Ancona, Italy. Phone: 39 71 5963467. Fax: 39 71 5963468. E-mail: cmalinf@popcsi.unian.it.

TABLE 1. MICs of cationic peptides and amikacin evaluated according to procedures outlined by the NCCLS and Hancock

Organisms (no. of strains)	Agent[b]	MIC (μg/ml)[a]					
		Range		50%		90%	
		NCCLS	Hancock	NCCLS	Hancock	NCCLS	Hancock
S. aureus (30)	BFII	2–64	0.5–8	8	2	32	8
	CP1	8–>128	4–128	64	16	>128	64
	IND	4–32	1–16	16	4	32	8
	MGII	4–128	1–64	16	8	128	32
	NS	2–64	1–16	16	4	32	8
	RNL	1–32	0.5–8	8	2	32	8
	AN	0.5–64	0.25–64	8	8	16	16
E. coli (30)	BFII	0.50–4	0.12–2	1	0.25	2	1
	CP1	0.12–4	0.12–2	1	0.25	4	1
	IND	0.5–8	0.25–8	4	2	8	8
	MGII	0.25–4	0.25–1	2	0.50	4	1
	NS	4–16	2–16	8	4	16	8
	RNL	1–32	0.25–32	8	4	32	16
	AN	0.06–8	0.06–8	2	2	8	8
P. aeruginosa (30)	BFII	2–128	0.50–16	16	4	32	8
	CP1	4–64	1–32	16	8	64	16
	IND	8–128	4–64	64	16	128	64
	MGII	2–128	0.50–32	16	8	128	32
	NS	16–128	4–128	64	32	128	128
	RNL	16–>128	8–128	64	32	>128	128
	AN	1–128	1–128	16	16	64	64

[a] 50% and 90%, MICs at which 50 and 90% of the strains are inhibited, respectively.
[b] BFII, buforin II; CP1, cecropin P1; IND, indolicidin; MGII, magainin II; NS, nisin; RNL, ranalexin; AN, amikacin.

significant. Drug concentrations required to inhibit 50 and 90% of the strains, as well as the ranges of the MICs of each agent, are listed in Table 1.

Finally, to obtain further data about effects produced by the binding of the peptides to the different plastic materials, two experiments were performed. First, the procedures outlined by the NCCLS were repeated by using polypropylene 96-well plates instead of polystyrene plates. Overall, this procedure produced MICs and MBCs that were twofold lower (data not shown). In these circumstances even amikacin showed a slight increase in activity (on the average, MICs and MBCs were 1.19-fold lower). Second, time-kill kinetics were determined by two different methods. The quality control strains *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were grown at 37°C in MH broth. Aliquots of exponentially growing bacteria were resuspended in fresh MH broth at approximately 10^7 cells/ml and exposed to each peptide (final concentration, 32 μg/ml) for 0, 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min at 37°C in two separate series of polystyrene or polypropylene test tubes. After these times, as well as in MBCs, samples were serially diluted in sodium HEPES buffer (10 mM, pH 7.2) to minimize the carryover effect. The diluted samples were plated onto MH agar plates to obtain viable colonies. Killing by all peptides in polypropylene tubes was shown to be the most rapid against the three control strains: the activity was complete after a mean 12.5-min exposure period (range, 5 to 40 min). On the contrary, killing in polystyrene tubes was complete after a mean 26.4-min exposure period (range, 10 to 90 min).

Vertebrate lytic peptides are known to have variable antibacterial, antifungal, and antiprotozoan activity in vitro. Nevertheless, there are few data on the broth microdilution methods and other in vitro susceptibility tests for cationic peptides (6, 8–10, 11, 14). In this study we compared the microtiter broth dilution method recommended by the NCCLS for bacteria that grow aerobically with the microtiter dilution method modified by Hancock for the cationic antimicrobial peptides. Statistical analysis showed that the differences between the two procedures were highly significant. The methods differ primarily for two reasons: the different plastic materials used and the different definition of the MIC. The effect of the plastic material was investigated by the time-dependent killing kinetics, which demonstrated a higher antimicrobial activity when polypropylene tubes were used. It has been demonstrated that the mechanism of activity of cationic peptides for both gram-positive and gram-negative bacteria is the interaction of the positively charged residues of the peptides with the negatively charged membranes of the organisms and, consequently, the formation of channels in the cytoplasmic membrane (10). Therefore, since many cationic peptides bind avidly to the negatively charged surface of several target cells and plastic materials, such as polystyrene microtiter plates, the use of inadequate techniques may involve the above-mentioned mode of action and underestimate their antimicrobial potency. Nevertheless, it remains difficult to evaluate data obtained by using the Hancock method and to compare them to results of conventional assays for established antimicrobial agents. Further in vitro studies are needed to standardize a reliable procedure to investigate the antimicrobial activity of the polycationic peptides.

REFERENCES

1. **Aley, S. B., M. Zimmerman, M. Hetsko, M. E. Selsted, and F. D. Gillin.** 1994. Killing of *Giardia lamblia* by cryptdins and cationic neutrophil peptides. Infect. Immun. **62:**5397–5403.
2. **Bevins, C. L., and M. Zasloff.** 1990. Peptides from frog skin. Annu. Rev. Biochem. **59:**395–414.
3. **Boman, H. G., and D. Hultmark.** 1987. Cell-free immunity in insects. Annu. Rev. Microbiol. **41:**103–126.
4. **Cannon, M.** 1987. A family of wound healers. Nature **328:**478.

5. **Clark, D. P., S. Durell, W. L. Maloy, and M. Zasloff.** 1994. Ranalexin: a novel antimicrobial peptide from bullfrog (*Rana catasbeiana*) skin, structurally related to the bacterial antibiotic polymyxin. J. Biol. Chem. **269:**10849–10855.
6. **De Lucca, A. J., and T. J. Walsh.** 1999. Antifungal peptides: novel therapeutic compounds against emerging pathogens. Antimicrob. Agents Chemother. **43:**1–11.
7. **Falla, T. J., and R. E. W. Hancock.** 1997. Improved activity of a synthetic indolicidin analog. Antimicrob. Agents Chemother. **41:**771–775.
8. **Giacometti, A., O. Cirioni, G. Greganti, M. Quarta, and G. Scalise.** 1998. In vitro activities of membrane-active peptides against gram-positive and gram-negative aerobic bacteria. Antimicrob. Agents Chemother. **42:**3320–3324.
9. **Hancock, R. E. W.** 1997. Antibacterial peptides and the outer membranes of gram-negative bacilli. J. Med. Microbiol. **46:**1–3.
10. **Hancock, R. E. W.** 1997. Peptides antibiotics. Lancet **349:**418–422.
11. **Hancock, R. E. W., and D. S. Chapple.** 1999. Peptide antibiotics. Antimicrob. Agents Chemother. **43:**1317–1323.
12. **Lee, J. Y., A. Boman, S. Chuanxin, M. Andersson, H. Jörnall, V. Mutt, and H. G. Boman.** 1989. Antimicrobial peptides from pig intestine: isolation of a mammalian cecropin. Proc. Natl. Acad. Sci. USA **86:**9159–9162.
13. **Lehrer, R. I., A. Barton, K. A. Daher, S. S. L. Harwig, T. Ganz, and M. E. Selsted.** 1989. Interaction of human defensins with *Escherichia coli*. J. Clin. Investig. **84:**553–561.
14. **Moore, A. J., W. D. Beazley, M. C. Bibby, and D. A. Devine.** 1996. Antimicrobial activity of cecropins. J. Antimicrob. Chemother. **37:**1077–1089.
15. **National Committee for Clinical Laboratory Standards.** 1993. Approved standards M7-A3. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards, Villanova, Pa.
16. **Vaara, M., and M. Porro.** 1996. Group of peptides that act synergistically with hydrophobic antibiotics against gram-negative enteric bacteria. Antimicrob. Agents Chemother. **40:**1801–1805.
17. **Wu, M., and R. E. W. Hancock.** 1999. Improved derivatives of bactenecin, a cyclic dodecameric antimicrobial cationic peptide. Antimicrob. Agents Chemother. **43:**1274–1276.
18. **Yi, G. S., C. B. Park, S. C. Kim, and C. Cheong.** 1996. Solution structure of an antimicrobial peptide buforin II. FEBS Lett. **398:**87–90.
19. **Zasloff, M.** 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA **84:**5449–5453.